

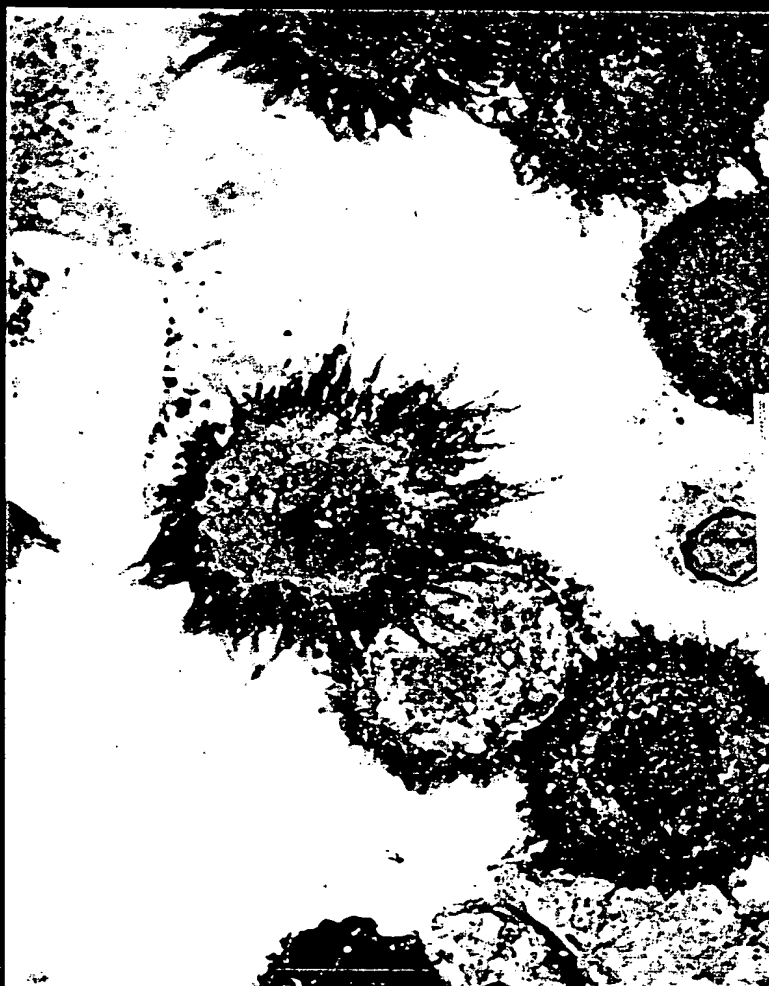
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The P2Y₁ receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel

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Summary. Recently we showed that the P2Y₁ receptor coupled to calcium mobilization is necessary to initiate ADP-induced human platelet aggregation. Since the thienopyridine compound clopidogrel specifically inhibits ADP-induced platelet aggregation, it was of interest to determine whether the P2Y₁ receptor was the target of this drug. Therefore we studied the effects of clopidogrel and of the two specific P2Y₁ antagonists A2P5P and A3P5P on ADP-induced platelet events in rats. Although clopidogrel treatment (50 mg/kg) greatly reduced platelet aggregation in response to ADP as compared to untreated platelets, some residual aggregation was still detectable. In contrast, A2P5P and A3P5P totally abolished ADP-induced shape change and aggregation in platelets from both control and clopidogrel-treated rats. A2P5P and A3P5P (100 µM) totally inhibited the [Ca²⁺]_i rise induced by ADP (0.1 µM) in control and clopidogrel-treated platelets, whereas clopidogrel treatment had no effect. Conversely, the inhibition of adenylyl

cyclase induced by ADP (5 µM) was completely blocked by clopidogrel but not modified by A2P5P or A3P5P (100 µM). A3P5P (1 mM) reduced the number of [³³P]2MeSADP binding sites on control rat platelets from 907 ± 50 to 611 ± 25 per platelet. After clopidogrel treatment, binding of [³³P]2MeSADP decreased to 505 ± 68 sites per platelet and further decreased to 55 ± 12 sites in the presence of A3P5P (1 mM). In summary, these results demonstrate that the platelet P2Y₁ receptor responsible for the initiation of aggregation in response to ADP is not the target of clopidogrel. Platelets may express another, as yet unidentified, P2Y receptor, specifically coupled to the inhibition of adenylyl cyclase and necessary to induce full platelet aggregation, which could be the target of this drug.

Keywords: P2T, antiplatelet drug, P2 receptor, [³³P]2MeSADP binding.

Nucleotide receptors, the so-called P2 receptors, are classified into two main families depending on their structure: the P2X family comprising 'ionotropic' or ligand-gated ion channel receptors and the P2Y family composed of G-protein coupled or 'metabotropic' receptors (North & Barnard, 1997). These families each contain several subtypes (P2Y₁ to P2Y₁₁ and P2X₁ to P2X₇) which differ in molecular structure and pharmacologic profile (North & Barnard, 1997; Communi *et al.*, 1998). P2 receptors are involved in many physiological processes including control of vascular tone, neurotransmission and platelet aggregation (Bhagwat & William, 1997). The central role of adenosine 5'-diphosphate (ADP) as an aggregating agent (Hellem,

1960; Gaardner *et al.*, 1961), not only in the physiological processes of haemostasis but also in the development and extension of arterial thrombosis (Maffrand *et al.*, 1988), has been long established and makes ADP receptors of potential clinical importance. Platelet activation by ADP leads to rapid calcium entry and mobilization of intracellular calcium stores (Heermiskerk & Sage, 1994), inhibition of adenylyl cyclase (Macfarlane, 1987), shape change from discoid to spherical form, and aggregation (Macfarlane, 1987; Hourani & Hall, 1996; Mills, 1996; Gachet *et al.*, 1997).

In view of the recent advances in our knowledge of the receptors mediating the effects of ADP on platelets, the P2T receptor should now be considered as a pharmacologic concept rather than a molecular entity (Gachet *et al.*, 1997). Thus, it has been demonstrated that platelets exhibit a functional P2X₁ receptor, activated by αβMeATP (MacKenzie *et al.*, 1996; Vial *et al.*, 1997), which is responsible for

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the rapid calcium entry induced by ADP but this receptor does not seem to be involved in ADP-induced platelet shape change or aggregation (Vial *et al.* 1997). since $\alpha\beta$ MeATP does not promote these responses and since desensitization of the P2X₁ receptor does not modify ADP-induced aggregation (Gachet *et al.* 1997). Several recent publications have reported detection of the P2X₁ receptor protein in platelets by immunoblot analyses (Scase *et al.* 1998; Sun *et al.* 1998; Clifford *et al.* 1998). The P2Y₁ receptor is also expressed by platelets (Léon *et al.* 1997; Jin *et al.* 1998). This receptor, when heterologously expressed in Jurkat cells, specifically couples to $[Ca^{2+}]_i$ mobilization and exhibits a pharmacological profile identical to that of the unknown platelet ADP receptor, ADP and related compounds being full agonists and ATP and its derivatives competitive antagonists (Léon *et al.* 1997; Hechler *et al.* 1998b). Furthermore, the fact that adenosine-2'-phosphate-5'-phosphate (A2P5P) and adenosine-3'-phosphate-5'-phosphate (A3P5P), which are specific P2Y₁ antagonists (Boyer *et al.* 1996), inhibit ADP-induced $[Ca^{2+}]_i$ increases, shape change and aggregation shows that the P2Y₁ receptor is responsible for these effects in platelets (Jin *et al.* 1998; Hechler *et al.* 1998a). On the other hand, the fact that under conditions where A2P5P and A3P5P totally abolish ADP-induced platelet aggregation and calcium movements, these molecules do not affect ADP-induced adenylyl cyclase inhibition, has led to the hypothesis of another P2 receptor coupled to adenylyl cyclase (Daniel *et al.* 1998; Hechler *et al.* 1998a).

The thienopyridine compound ticlopidine and its derivative clopidogrel, two specific and potent inhibitors of ADP-induced platelet aggregation, are used clinically as anti-thrombotic drugs (Schrör, 1993). Ticlopidine and clopidogrel are nevertheless inactive *in vitro* and must be metabolized in the liver in order to acquire their anti-aggregatory properties (Herbert *et al.* 1993). These compounds inhibit ADP-induced aggregation of human and rat platelets, the effect of ADP on adenylyl cyclase in human (Gachet *et al.* 1990), rat and rabbit platelets (Defreyn *et al.* 1991) and G-protein activation (Gachet *et al.* 1992), whereas they do not block ADP-induced platelet shape change (Gachet *et al.* 1995) or calcium movements (Gachet *et al.* 1990). Clopidogrel also irreversibly reduces the number of binding sites for radiolabelled 2-methylthio-adenosine 5'-diphosphate (2MeSADP) on human (Mills *et al.* 1992) or rat platelets (Savi *et al.* 1994; Gachet *et al.* 1995) by approximately 70%, leaving the residual sites insensitive to treatment. However, the precise mechanism of action of thienopyridines is still unknown and the molecular target of these drugs has not yet been identified.

In the present work we addressed the question of whether the P2Y₁ receptor could be the target of clopidogrel, by studying the effects of this drug and of A2P5P and A3P5P on ADP-induced platelet events.

MATERIALS AND METHODS

Materials. Adenosine 5'-O-(1-thiotriphosphate) (Sp-isomer) (Sp-ATP α S) was from Boehringer (Mannheim, Germany). 2MeSADP and propranolol were from Research

Biochemicals Incorporated (Natick, U.S.A.) and ADP, A2P5P, A3P5P, thrombin, prostaglandin E₁ (PGE₁), adrenaline and essentially fatty acid free human serum albumin from Sigma (Saint Quentin-Fallavier, France). Fura-2/acetoxymethyl ester (fura-2/AM) was purchased from Calbiochem (Meudon, France) and the cyclic adenosine 3'-5'-monophosphate (cAMP) assay kit from Amersham (Les Ulis, France). Apyrase was purified from potatoes as previously described (Cazenave *et al.* 1983). Clopidogrel was provided by Sanofi Recherche (Toulouse, France) and [³²P]2MeSADP (31.45 MBq/mmol) by Du Pont NEN® (Le Blanc Mesnil, France) and the radioligand was found to be 97.3% pure. A2P5P and A3P5P were checked for purity by high-performance liquid chromatography (HPLC) analysis on a Partisil 10 μ SAX column (Interchrom, Interchim, Montluçon, France) eluted with a linear gradient of 0–1 M ammonium phosphate buffer, pH 3.8.

Platelet aggregation studies. Male Wistar rats aged 7–10 weeks and weighing 200–250 g were used in groups of five animals. Clopidogrel was solubilized in water and arabic gum and force-fed to the rats twice at a dose of 50 mg/kg, 24 h and 2 h before blood collection, whereas control rats received water and arabic gum in the same manner. This dose of 50 mg/kg is known to induce the maximal inhibitory effect of clopidogrel (Gachet *et al.* 1995). Blood was drawn from the abdominal aorta under Rompun (0.2 ml/kg) and Imalgene 1000 (1 ml/kg) anaesthesia, anticoagulated with sodium citrate (3.15%) and centrifuged at 1570 *g* for 2 min to obtain citrated platelet-rich plasma (cPRP). Platelet aggregation was measured in cPRP from control and clopidogrel-treated rats by a turbidimetric method at 37°C in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). A 450 μ l aliquot of platelet suspension was stirred at 1100 r.p.m. and activated by addition of different agonists, in the presence of control buffer or A2P5P or A3P5P at varying concentrations, in a final total volume of 500 μ l. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline.

Preparation of washed rat platelets. Blood anticoagulated with acid-citrate-dextrose solution (1 volume of ACD for 6 blood volumes) was centrifuged at 1570 *g* for 2 min at 37°C, after which the upper PRP phase was removed and centrifuged at 1570 *g* for 15 min at 37°C. The platelet pellet was washed twice in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, 5 mM Hepes, pH 7.3) containing 0.35% human serum albumin as previously described (Cazenave *et al.* 1983) and finally resuspended at 3×10^5 platelets/ μ l in the same buffer, in the presence of 0.02 U/ml of the ADP scavenger apyrase (adenosine 5'-triphosphate diphosphohydrolase, EC 3.6.1.5), a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. This preparation of washed platelets was kept at 37°C and used for adenylyl cyclase and binding experiments.

$[Ca^{2+}]_i$ measurements. After the first wash, the platelet pellet was resuspended in Tyrode's buffer containing 0.35% human serum albumin but no calcium, at a concentration of about 7×10^5 platelets/ μ l. Platelets were loaded with 15 μ M

fura-2/AM for 45 min at room temperature in the dark, in the presence of 0.1 mM aspirin to prevent platelet activation through synthesis of thromboxane A_2 . The suspension was then centrifuged at 1570 *g* for 8 min and finally resuspended at room temperature at a concentration of 2×10^5 platelets/ μ l, in Tyrode's buffer containing 0.02 U/ml apyrase and 0.1% essentially fatty acid free human serum albumin but no calcium. Aliquots of fura-2-loaded control or clopidogrel-treated rat platelets were transferred to a 10 \times 10 mm quartz cuvette maintained at 37°C and fluorescence measurements were performed under continuous stirring, using a PTI Deltascan spectrophluorimeter (Photon Technology International Inc., Princetown, N.J., U.S.A.). The excitation wavelength was alternately fixed at 340 or 380 nm, fluorescence emission was determined at 510 nm and results were calculated as the fluorescence ratio (340/380 nm).

Measurement of adenylyl cyclase activity. A 450 μ l aliquot of washed platelets from control or clopidogrel-treated rats was stirred at 1100 r.p.m. in an aggregometer cuvette and the following reagents were added at 30 s intervals: 10 μ M $CaCl_2$, 100 μ M A2P5P, A3P5P or ATP α S and 5 μ M ADP or vehicle (Tyrode's buffer containing no Ca^{2+} or Mg^{2+}). After 1 min the reaction was stopped by addition of 50 μ l ice-cold 6 \times N perchloric acid. Perchloric acid extracts were centrifuged at 11 000 *g* for 5 min to eliminate protein precipitate and cyclic AMP was isolated from the supernatants as described by Khym (1975) using a mixture of trioctylamine and heion (28/22, v/v). The upper aqueous phase was lyophilized and the dry residue dissolved in the buffer provided with the commercial radioimmunoassay kit for cyclic AMP.

[^{33}P]2MeSADP binding. 2MeSADP, an analogue of ADP substituted at position 2 of the purine ring, is a more potent (about 10-fold) platelet aggregating agent than ADP (Mills

1996). Binding of [^{33}P]2MeSADP to washed platelets from control or clopidogrel-treated rats was determined by incubation of [^{33}P]2MeSADP (0.1 nM, 200 000 cpm) with washed platelet suspensions (3×10^5 platelets/ μ l) for 5 min at 37°C, in the presence or absence of 1 mM A3P5P, in a final volume of 1 ml in 3 ml polypropylene tubes. Experiments were started by addition of washed platelets to the reaction mixture and carried out in triplicate. The reaction was terminated by dilution with 2 ml of buffer and rapid filtration through Whatman GF/C glass filters under vacuum, after which the tubes and filters were rinsed twice. Radioactivity of the platelets on the filters was measured by scintillation counting (Wallac 1409 counter, Turku, Finland). Non-specific binding, determined by incubation in the presence of 1 μ M unlabelled 2MeSADP, amounted to about 5% of the total binding. Saturation and displacement experiments were performed using a single concentration of [^{33}P]2MeSADP (0.1 nM) and increasing concentrations of appropriate unlabelled ligands.

Data analysis. The concentration producing 50% of the maximal effect (EC_{50}) and the apparent dissociation constants of inhibitors (pA_2) were calculated using the GraphPad software package (GraphPad, San Diego, Calif., U.S.A.). Binding data were analysed and plotted with the LIGAND program (Munson & Rodbard, 1980).

RESULTS

Platelet aggregation studies

ADP-induced aggregation in cPRP of platelets from clopidogrel-treated rats was greatly reduced as compared to control platelet aggregation (Fig 1A). However, at this dose (50 mg/kg), there persisted a residual aggregation response

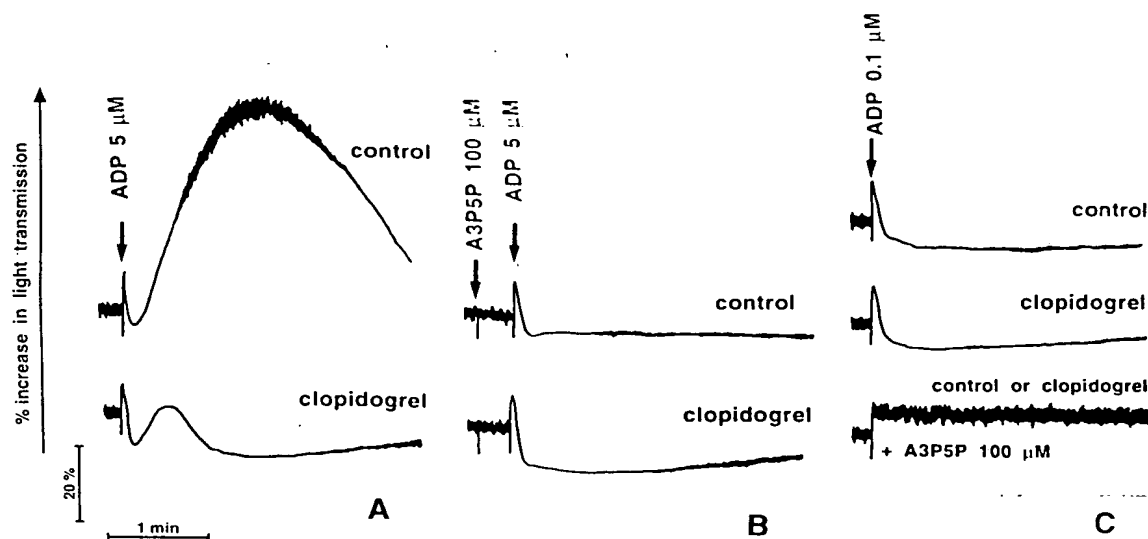


Fig 1. Effects of A3P5P on control and clopidogrel (50 mg/kg) treated rat platelets. (A) Aggregation in cPRP of platelets from control or clopidogrel-treated rats in response to 5 μ M ADP. (B) Aggregation induced by 5 μ M ADP was inhibited by 100 μ M A3P5P. (C) Shape change representative of five independent experiments giving identical results.

to 5 μM ADP. As already observed in previous work (Gachet *et al.* 1990, 1995), clopidogrel did not inhibit the shape change induced by 0.1 μM ADP (data not shown). This is the primary morphological change of platelets which is reflected in a characteristic decrease in light transmission in the aggregometer. The adenine nucleotide derivatives A2P5P and A3P5P did not induce platelet shape change or aggregation, even at high concentrations (up to 100 μM). On the other hand, these two specific P2Y₁ receptor antagonists inhibited ADP-induced aggregation (Fig 1B) and likewise the shape change induced by 0.1 μM ADP (Fig 1C), in control or clopidogrel-treated rat platelets. A3P5P produced a parallel concentration-dependent shift to the right of the dose-response curve for ADP (Fig 2) in control platelets. EC₅₀ values for ADP-induced platelet aggregation were $1.6 \pm 0.2 \mu\text{M}$, $2.2 \pm 0.4 \mu\text{M}$, $2.6 \pm 0.7 \mu\text{M}$, $6.0 \pm 1.7 \mu\text{M}$, $11.7 \pm 2.8 \mu\text{M}$ and $19.2 \pm 2.1 \mu\text{M}$ in the presence of 0, 1, 3, 10, 30 and 100 μM A3P5P respectively. Schild analysis of the data resulted in a pA₂ value of 5.4 and a slope of 0.69 ± 0.05 , which suggests that the antagonism by A3P5P of ADP-induced rat platelet aggregation is non-competitive. The isomer A2P5P produced a similar right-hand shift of the dose-response curve for ADP and Schild analysis of the inhibition gave a pA₂ value of 5.6 and a slope of 0.74 ± 0.03 , also suggesting non-competitive antagonism.

[Ca²⁺]_i measurements

The ADP-induced intracellular calcium rise was not modified by clopidogrel treatment, in the presence (Figs 3A and 3C) or absence (Figs 3B and 3D) of 2 mM external calcium.

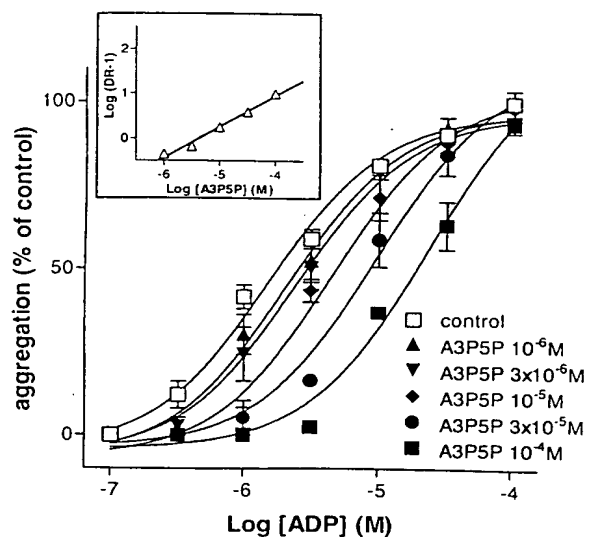


Fig 2. Inhibition by A3P5P of ADP-induced aggregation of control rat platelets. Aggregation was induced by increasing concentrations of ADP, alone or in the presence of increasing concentrations of A3P5P added 30 s before ADP. Inset: Schild regression analysis of the dose-response curve for ADP. Curves represent the mean of four independent experiments and bars show the SEM.

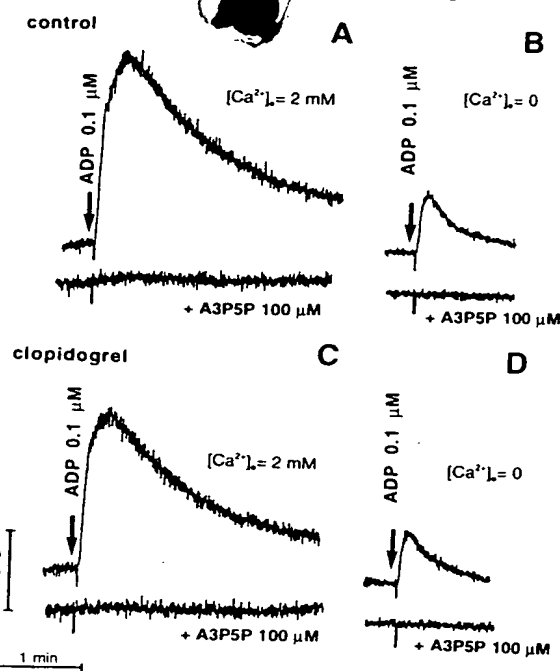


Fig 3. Effects of A3P5P (100 μM) and clopidogrel treatment (50 mg/kg) on [Ca²⁺]_i rises induced by 0.1 μM ADP. 100 μM A3P5P, added 30 s before ADP, totally abolished ADP-induced [Ca²⁺]_i increases in control (A, B) and clopidogrel-treated (C, D) rat platelets, in the presence of 2 mM external calcium (A, C) or in the absence of external calcium (0.2 mM EGTA) (B, D). Data are from one experiment representative of three independent experiments giving identical results.

Conversely, 100 μM A3P5P totally inhibited the [Ca²⁺]_i increases induced by 0.1 μM ADP, a concentration close to its EC₅₀ for this effect, in control or clopidogrel-treated platelets resuspended in Tyrode's buffer containing 0.35% human albumin and either 2 mM calcium (Figs 3A and 3C) or no calcium (0.2 mM EGTA) (Figs 3B and 3D). Identical inhibition of ADP-induced [Ca²⁺]_i rises was obtained using A2P5P (data not shown). These results suggest that platelet aggregation in response to ADP depended on the mobilization of internal calcium stores through activation of the P2Y₁ receptor and that this receptor was probably not the target of clopidogrel.

Adenyl cyclase activity

A2P5P and A3P5P (100 μM) had no influence on basal levels of cyclic AMP in rat platelets or on the cyclic AMP levels induced by 10 μM PGE₁ (data not shown). The inhibition by 5 μM ADP of PGE₁-stimulated cyclic AMP levels in control rat platelets was not reversed in the presence of 100 μM A3P5P or A2P5P, in contrast to 100 μM Sp-ATP α S, which totally reversed the effects of ADP (Fig 4, left panel). Sp-ATP α S is a well known antagonist of the ADP receptor affecting ADP-induced platelet aggregation, intracellular calcium increases

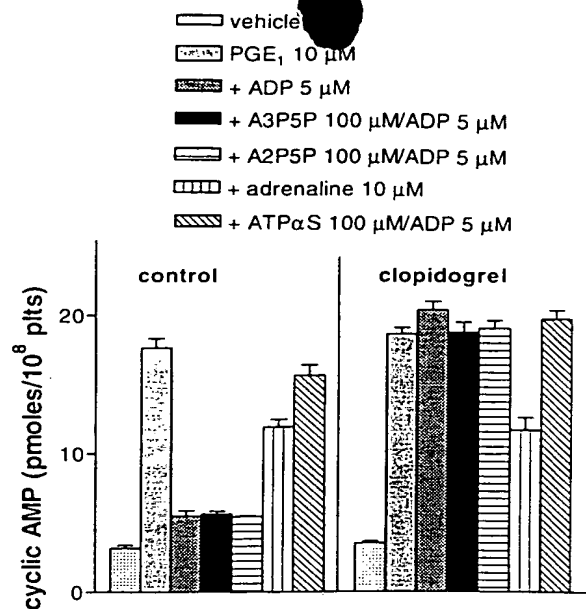


Fig 4. Cyclic AMP measurements in control (left panel) and clopidogrel (50 mg/kg) treated (right panel) washed rat platelets. Data are mean values (\pm SEM) from three separate experiments each performed in triplicate. When adrenaline was tested, the β -adrenergic specific antagonist propranolol (10 μ M) was added to avoid the β -adrenergic coupled stimulatory effect of adrenaline on adenylyl cyclase.

and adenylyl cyclase inhibition (Hourani & Hall, 1996; Mills, 1996). The α_2 coupled cyclase inhibitor adrenaline (10 μ M) was also able to inhibit PGE₁-stimulated cyclic AMP levels, provided its β -adrenergic coupled stimulatory effect on adenylyl cyclase was prevented by addition of the β -adrenergic-specific antagonist propranolol (10 μ M) (Fig 4, left panel).

ADP-induced adenylyl cyclase inhibition was, in contrast, completely abolished by clopidogrel treatment (Fig 4, right panel). This effect was specific, since 10 μ M adrenaline still inhibited PGE₁-stimulated cyclic AMP levels, which confirmed earlier observations that clopidogrel is a specific antagonist of ADP-induced inhibition of the adenylyl cyclase pathway.

Potentiality by adrenaline of ADP-induced platelet aggregation

The aggregation of control or clopidogrel-treated rat platelets induced by 0.25 μ M ADP (Fig 5A, top and bottom, respectively) was potentiated in the presence of 10 μ M adrenaline (Fig 5C, top and bottom, respectively). However, under conditions where 100 μ M A3P5P totally inhibited ADP-induced aggregation (Fig 5B, top and bottom, respectively), no potentiation by adrenaline could be detected in either control or clopidogrel-treated platelets (Fig 5D, top and bottom, respectively), suggesting that the P2Y₁ receptor is necessary for the initiation of aggregation in response to ADP.

[³²P]2MeSADP binding

Saturation experiments using control rat platelets revealed 907 ± 50 [³²P]2MeSADP binding sites per platelet with an

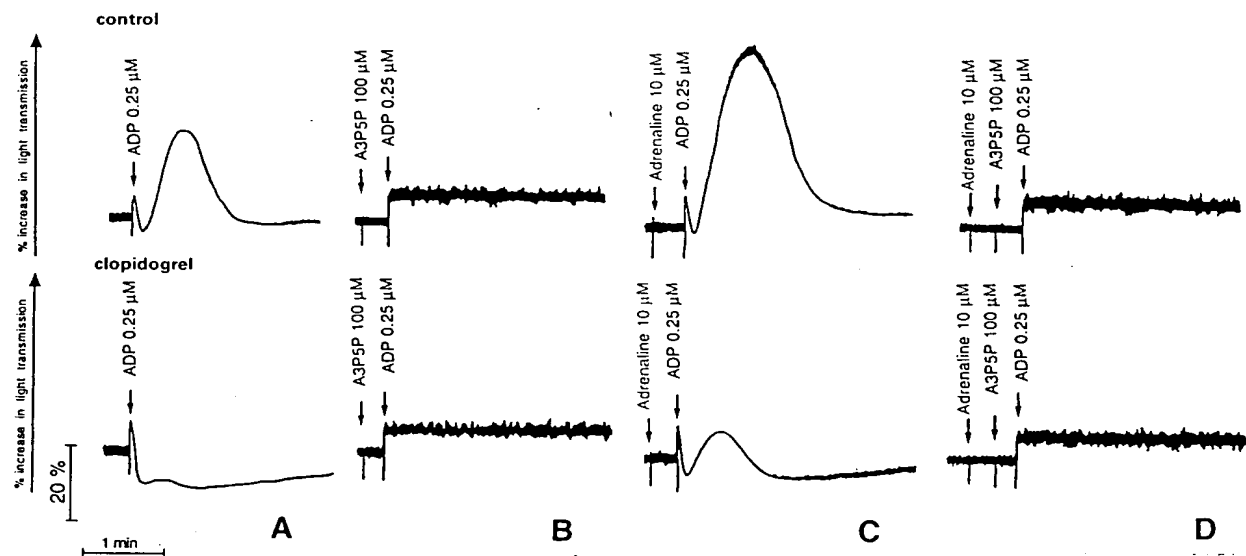


Fig 5. Potentiating effect of adrenaline on ADP-induced aggregation of platelets from control or clopidogrel-treated rats. Aggregation induced by 0.25 μ M ADP (A) was potentiated in the presence of 10 μ M adrenaline (C). This aggregation was inhibited by 100 μ M A3P5P in the absence (B) or presence (D) of 10 μ M adrenaline. Experiments were performed in the presence of the β -adrenergic-specific antagonist propranolol (10 μ M) to avoid the β -adrenergic coupled stimulatory effect of adrenaline on adenylyl cyclase. Results are from one experiment representative of three independent experiments giving identical results.

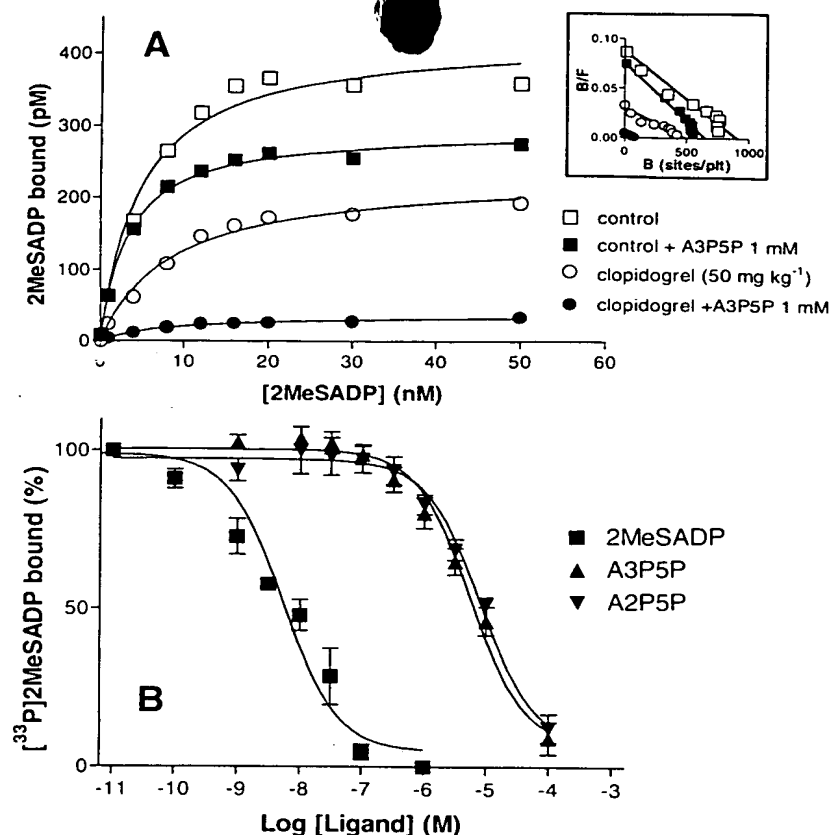


Fig 6. Binding of [³³P]2MeSADP to control and clopidogrel (50 mg/kg) treated washed rat platelets. (A) Equilibrium specific binding of [³³P]2MeSADP to control and clopidogrel-treated rat platelets in the presence or absence of 1 mM A3P5P. Data are mean values from one experiment performed in triplicate and are representative of four separate experiments giving identical results. (B) Competition curves for [³³P]2MeSADP binding to clopidogrel-treated rat platelets. Residual binding of [³³P]2MeSADP (0.1 nM) to clopidogrel-treated platelets was displaced by increasing concentrations of unlabelled 2MeSADP ($K_i = 8 \pm 1.3$ nM), A2P5P ($K_i = 9.5 \pm 1.1$ μ M) or A3P5P ($K_i = 8.7 \pm 1.2$ μ M). Points represent the mean values of nine data points.

affinity of 4.5 ± 0.3 nM (Fig 6A). In the presence of 1 mM A3P5P, the number of [³³P]2MeSADP binding sites decreased to 611 ± 25 per platelet and these residual sites displayed an affinity of 3.5 ± 0.2 nM. After clopidogrel treatment, binding of [³³P]2MeSADP was reduced by 50% as compared to control rat platelets, the remaining 505 ± 68 'clopidogrel insensitive' sites having an affinity of 8.3 ± 0.1 nM. Finally, in the presence of 1 mM A3P5P, [³³P]2MeSADP binding to clopidogrel-treated platelets fell to 55 ± 12 sites per platelet with an affinity of 5.9 ± 1.1 nM.

The residual binding of [³³P]2MeSADP to platelets from clopidogrel-treated rats was displaced by increasing concentrations of A2P5P ($K_i = 9.5 \pm 1.1$ μ M) or A3P5P ($K_i = 8.7 \pm 1.2$ μ M) and likewise by unlabelled 2MeSADP ($K_i = 8 \pm 1.3$ nM) (Fig 6B). These results suggest that the 'clopidogrel insensitive' sites could correspond to the P2Y₁ receptor.

DISCUSSION

In a recent study we demonstrated that the P2Y₁ receptor was necessary to initiate ADP-induced aggregation of human platelets (Hechler *et al.* 1998a), thus confirming and extending previous work which had shown the P2Y₁ receptor to be an ADP receptor present in platelets (Léon *et al.* 1997; Hechler *et al.* 1998b) and responsible for ADP-induced calcium mobilization and platelet shape change (Jin

et al. 1998). Since the thienopyridine compound clopidogrel specifically inhibits ADP-induced platelet aggregation, it was of further interest to determine whether the P2Y₁ receptor could be the target of this drug. Therefore in the present work we studied the effects of clopidogrel and of the two specific P2Y₁ antagonists A2P5P and A3P5P on ADP-induced platelet events.

When platelet aggregation was measured in citrated plasma, clopidogrel treatment led to an important reduction in ADP-induced aggregation as compared to untreated platelets, but a residual response was still detectable. These results confirm earlier observations that although clopidogrel is a specific inhibitor of ADP-induced platelet aggregation, part of the response to ADP is insensitive to this drug and in particular platelet shape change (Gachet *et al.* 1997). In contrast, ADP-induced shape change and aggregation of control rat platelets were totally inhibited by the two specific P2Y₁ antagonists A2P5P and A3P5P. This antagonism was selective, as A2P5P and A3P5P (100 μ M) did not affect platelet aggregation induced by thrombin (0.1 U/ml) under conditions where ADP secreted from platelet-dense granules was removed by addition of apyrase (0.2 U/ml) (data not shown). These results provide further evidence that the P2Y₁ receptor is necessary for the initiation of aggregation in response to ADP, and the fact that the inhibition by A2P5P and A3P5P was non-competitive reinforces the hypothesis

that ADP-induced aggregation may involve more than one receptor. In addition, since residual aggregation of clopidogrel-treated rat platelets in response to ADP was completely inhibited by the two P2Y₁ antagonists, this receptor does not appear to be the target of clopidogrel.

At the intracellular level, A2P5P and A3P5P totally inhibited ADP-induced [Ca²⁺]_i mobilization but had no effect on ADP-induced inhibition of adenylyl cyclase. This supports the hypothesis that initiation of aggregation in response to ADP is dependent on mobilization of internal calcium stores and inhibition of adenylyl cyclase cannot alone promote aggregation (Haslam, 1973; Hechler *et al.* 1998a). The mechanism by which ADP induces the mobilization of intracellular calcium stores in platelets has been poorly understood due to controversy concerning the ability of ADP to activate phospholipase C (PLC) (Hourani & Hall, 1996; Mills, 1996; Gachet *et al.* 1997). Several studies suggested that this process could be independent of PLC activation (Vickers *et al.* 1990; Raha *et al.* 1993), whereas others found some inositol-1,4,5-triphosphate (IP3) formation in response to ADP stimulation (Daniel *et al.* 1986, 1998). Recently it has been possible to obtain knock-out mice lacking the gene

coding for the α subunit of the Gq protein (Offermans *et al.* 1997). Platelets from these mice did not aggregate in response to ADP under conditions where IP3 generation was totally abolished, which suggests that the PLC pathway is necessary for ADP stimulation to raise [Ca²⁺]_i and that this [Ca²⁺]_i increase is essential to platelet aggregation. However, since clopidogrel has no effect on the [Ca²⁺]_i rise induced by ADP but nevertheless strongly inhibits aggregation, an increase in [Ca²⁺]_i cannot solely account for platelet aggregation and inhibition of adenylyl cyclase, which is totally blocked under clopidogrel treatment, may be necessary to sustain a full aggregation response. Thus, the adenylyl cyclase pathway would potentiate the aggregation initiated by agonists of the P2Y₁ receptor. This potentiation of aggregation by inhibition of adenylyl cyclase is clearly demonstrated by the fact that adrenaline, which is specifically coupled through platelet α_2 -adrenergic receptors to adenylyl cyclase inhibition in the absence of any increase in [Ca²⁺]_i, does not itself induce platelet aggregation (Lanza *et al.* 1988) but potentiates the aggregation induced by ADP in control or clopidogrel-treated platelets. As no potentiation could be detected in the presence of 100 μ M A3P5P, a

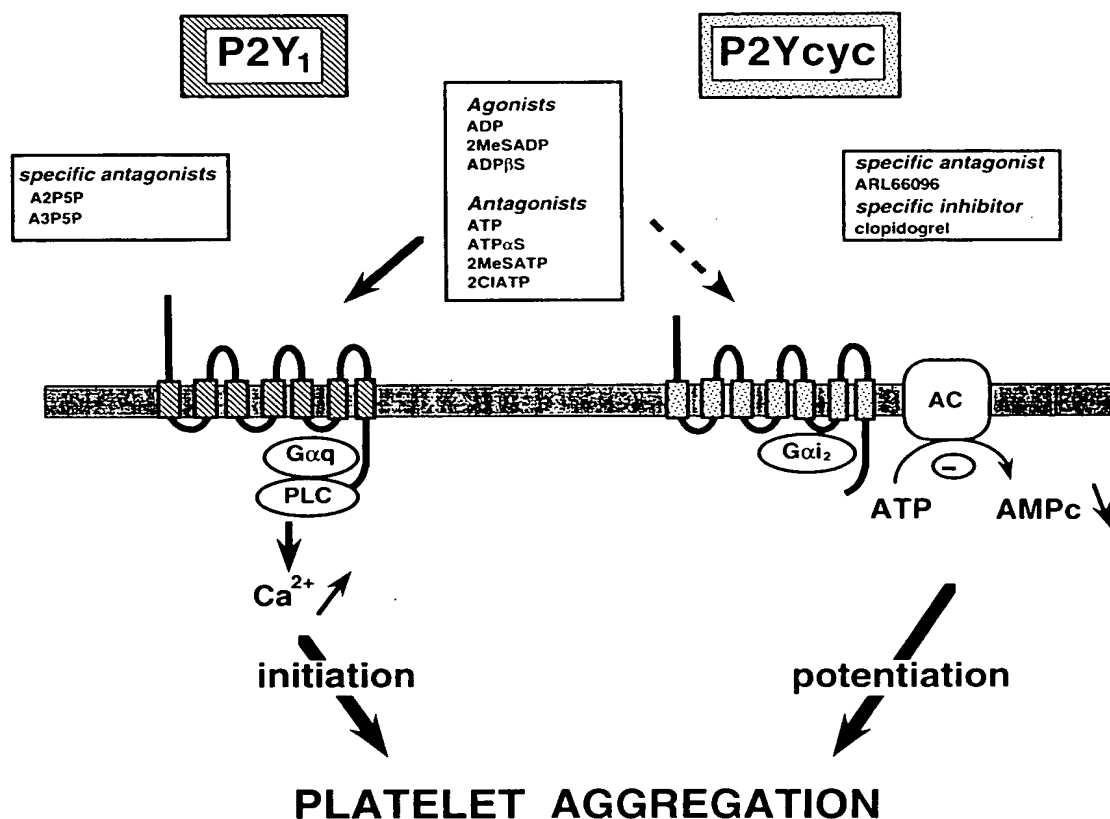


Fig 7. Current model of the mechanism of ADP-induced platelet aggregation. ADP initiates platelet aggregation through activation of the P2Y₁ receptor specifically coupled to the mobilization of internal calcium stores. Another, as yet unidentified, P2Y receptor, which we propose to call 'P2Y_{cyc}', specifically coupled to the inhibition of adenylyl cyclase, may potentiate the aggregation initiated by activation of P2Y₁. Specific antagonists of the P2Y₁ receptor include A2P5P and A3P5P. ARL66096 is a specific antagonist of the ADP-induced adenylyl cyclase pathway, whereas clopidogrel is a specific inhibitor and is used clinically as an antithrombotic drug.

concentration which totally abolishes ADP-induced aggregation of platelets from both control and clopidogrel-treated rats. Inhibition of adenylyl cyclase is clearly not sufficient to trigger this process. Therefore activation of the G_i pathway is clearly involved in the potentiation of platelet responses to ADP. However, since ADP does not induce a decrease in cyclic AMP in the course of aggregation, cyclic AMP levels cannot be directly responsible for this effect and further investigations will be required to define the mechanism of potentiation.

Binding experiments were performed to determine whether the P2Y₁ receptor was the molecular target of clopidogrel. Previous experiments using clopidogrel-treated rat platelets had distinguished two populations of 2MeSADP binding sites, 'clopidogrel sensitive' and 'insensitive' sites representing respectively 70% and 30% of the total binding sites (Savi *et al.* 1994; Gachet *et al.* 1995). In the present work we found that A3P5P inhibition led to a 30% reduction in the number of [³³P]2MeSADP binding sites on control platelets, whereas the sites insensitive to clopidogrel treatment amounted to about 50% of the initial binding sites present on control platelets. The proportion of clopidogrel-insensitive sites observed in these experiments was greater than in previous studies (Savi *et al.* 1994; Gachet *et al.* 1995). This discrepancy may be due to the fact that the earlier experiments were performed in female rats, whereas the present results were obtained in males, which have been shown to be less sensitive to clopidogrel treatment than females, even at high doses (Herbert *et al.* 1993). A3P5P almost totally abolished binding of [³³P]2MeSADP to the residual sites on clopidogrel-treated rat platelets, suggesting that the P2Y₁ receptor could represent about 30–50% of the total platelet ADP receptors and in fact correspond to the clopidogrel-insensitive binding sites.

The present study demonstrated that the target of clopidogrel is not the P2Y₁ receptor but the ADP-induced adenylyl cyclase pathway, thus reinforcing the hypothesis of the existence of a receptor distinct from P2Y₁ and specifically coupled to the inhibition of adenylyl cyclase. Although the P2Y₁ receptor would appear to be responsible for the initiation of aggregation, this receptor coupled to adenylyl cyclase, which we propose to call 'P2Y₁cyc', could mediate its further amplification (Fig 7). Identical conclusions were recently drawn using another P2Y₁ antagonist, A3P5PS, in rabbit platelets (Savi *et al.* 1998) and using A2P5P and A3P5P in human platelets (Hechler *et al.* 1998a; Jin *et al.* 1998; Daniel *et al.* 1998).

Daniel *et al.* (1998) have shown that ARL66096, like clopidogrel, is a specific and potent inhibitor of ADP-induced human platelet aggregation (Humphries *et al.* 1994), which reverses ADP-induced adenylyl cyclase inhibition but fails to inhibit ADP-induced platelet shape change, [Ca²⁺]_i mobilization or IP₃ production (Daniel *et al.* 1998). It is well established that ADP, which is stored at very high concentrations in platelet-dense granules, contributes to and reinforces aggregation in response to agents inducing dense granule secretion and hence plays a pivotal role in thrombosis (Maffrand *et al.* 1988). Clopidogrel inhibits the response to low concentrations of all aggregating agents

triggering platelet-dense granule secretion by blocking amplification of the aggregation process by released ADP. This drug has been found to be efficient in preventing the occurrence of thrombotic events in several cardiovascular pathologies (Herbert *et al.* 1993). ARL67085, a compound from the same series as ARL66096 (Humphries *et al.* 1995b), has shown antithrombotic activity in rat models and is under clinical investigation (Humphries *et al.* 1995a; Williams, 1996). It can be speculated that the potent antithrombotic effects of all these compounds originate from their inhibition of the ADP-induced adenylyl cyclase pathway.

In conclusion, our results show that the P2Y₁ receptor responsible for the mobilization of intracellular calcium stores and the initiation of platelet aggregation in response to ADP is not the target of clopidogrel. This target, which should be a P2Y receptor specifically coupled to adenylyl cyclase inhibition and responsible for amplification of the response initiated through P2Y₁, remains to be identified.

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